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Simvastatin Prevents Skeletal Metastasis of Breast Cancer by an Antagonistic Interplay between p53 and CD44*[S]

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Substantial data from clinical trials and epidemiological studies show promising results for use of statins in many cancers, including mammary carcinoma. Breast tumor primarily metastasizes to bone to form osteolytic lesions, causing severe pain and pathological fracture. Here, we report that simvastatin acts as an inhibitor of osteolysis in a mouse model of breast cancer skeletal metastasis of human mammary cancer cell MDA-MB-231, which expresses the mutant p53R280K. Simvastatin and lovastatin attenuated migration and invasion of MDA-MB-231 and BT-20 breast tumor cells in culture. Acquisition of phenotype to express the cancer stem cell marker, CD44, leads to invasive potential of the tumor cells. Interestingly, statins significantly decreased the expression of CD44 protein via a transcriptional mechanism. shRNA-mediated down-regulation of CD44 markedly reduced the migration and invasion of breast cancer cells in culture. We identified that in the MDA-MB-231 cells, simvastatin elevated the levels of mutated p53R280K, which was remarkably active as a transcription factor. shRNA-derived inhibition of mutant p53R280K augmented the expression of CD44, leading to increased migration and invasion. Finally, we demonstrate an inverse correlation between expression of p53 and CD44 in the tumors of mice that received simvastatin. Our results reveal a unique function of statins, which foster enhanced expression of mutant p53R280K to prevent breast cancer cell metastasis to bone.

Statins are potent inhibitors of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the mevalonate pathway for the biosynthesis of cholesterol (1). These compounds have been used for decades as safe and effective drugs in the control of hypercholesterolemia. The mevalonate pathway also produces a number of important end products, which include isoprenoid precursors, ubiquinone, dolichol, and isopentenyladenine (2). Statins also show anti-carcinogenic effects in rodent models of lung, prostate, melanoma, colon, glioma, and mammary tumorigenesis, and beneficial effects of statins have been seen in different cancers, including breast cancer (2, 3). For example, a significant 20% reduction in overall cancer risk was observed in patients with statin use (4). A recent study conducted in women who used statins showed significantly reduced risk of breast cancer as compared with nonusers (5). Moreover, an independent study of women who used statins for more than 4 years reported a significantly lower risk of breast cancer in this group (6).

Several distinct mechanisms have been proposed whereby statins block tumor cell proliferation and induce apoptosis. For example, inhibition of geranylgeranyl pyrophosphate and farnesyl pyrophosphate production by statins prevents the post-translational modification of Rho and Ras GTPases necessary for their membrane localization (2). Rho proteins regulate the proliferative and invasive potential of various tumor cells, including breast cancer cells (2). Thus, suppression of geranylgeranylation of RhoA by statins may significantly inhibit tumor cell growth. Additionally, by modulating the Ras GTPase function, statins inhibit the MAPK cascade and CDK2 activity, which may affect the degradation of p21 and p27 cyclin kinase inhibitors that regulate tumor cell proliferation and apoptosis (7–9). Apart from these signal transduction mechanisms, we have recently reported that simvastatin attenuates the Akt kinase activity in the tumor xenografts of breast cancer cells in mice (10). We identified increased expression of the tumor suppressor protein phosphatase and tensin homolog deleted in chromosome ten (PTEN) as the potential mechanism (10). Furthermore, we established a role for NFκB in the derepression of PTEN expression and the anti-apoptotic Bcl_{XL} expression, both of which contribute to proliferation and apoptosis of breast cancer cells *in vitro* in culture, and in tumor xenografts (10).

Although stage I breast cancer patients show 98% 5-year relapse-free survival rate, basal breast carcinomas relapse significantly earlier than luminal breast cancers (11, 12). Mortality in cancer, including breast cancer, is mainly dependent on the acquisition of metastatic phenotype of the cancer cells. Invasive power is controlled by many genetic and epigenetic changes that occur in the tumor cells. In fact, a dormant epithelial-mes-

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enchymal trans-differentiation program, sufficient to execute most of the steps of metastatic cascade, may be activated in a single step. The invasion-metastatic process is initiated by local invasion, called intravasation, followed by survival and translocation through the blood and lymphatic vessels leading to extravasation, formation of micrometastasis, and finally colonization (macrometastasis) of the organ (13).

The metastatic potential of tumor cells for different organs may be regulated by specific gene expression profiles inherent in the cancer cells as well as by the structure of the target organ. Thus, breast adenocarcinoma predominantly metastasizes to lung, liver, brain, and bone, and expression of specific marker proteins has been reported for lung-specific extravasation of breast cancer cells (14–16). The composition of capillary wall and subadjacent parenchyma varies significantly among different organs, and this may impact tumor infiltration. For example, the bone marrow sinusoid capillaries are fenestrated and permit increased trafficking of hematopoietic cells (17). On the other hand, lung capillaries are composed of endothelial lining surrounded by basement membrane and alveolar cells, which pose an obstacle to the circulating tumor cells, unless they express genes for transendothelial migration. Therefore, the bone marrow sinusoid capillaries are extremely permissive for the disseminated breast tumor cells in the circulation. Breast cancer metastasis to bone typically results in osteolytic lesions, which involve mobilization of the osteoclasts causing bone resorption with nerve compression, bone fracture, hypercalcemia, and severe pain (18). In this study, we show that simvastatin markedly prevents human MDA-MB-231 breast cancer cell metastasis to bone and inhibits migration and invasion of these cells *in vitro*. Simvastatin suppresses expression of the stem cell marker CD44 necessary for migration by increasing expression of mutant p53 in MDA-MB-231 cells. Moreover, in MDA-MB-231 mice xenografts, simvastatin increases expression of p53 with concomitant reduction in CD44 expression.

EXPERIMENTAL PROCEDURES

Reagents—MDA-MB-231 human mammary carcinoma cells were obtained from American Type Culture Collection. BT-20 human breast cancer cells were kind gift from Prof. Lu Zhe Sun (Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio). These cells were grown in DMEM supplemented with penicillin, streptomycin, and 10% fetal bovine serum. Simvastatin and lovastatin were purchased from LKT Laboratories Inc. Trans-well chambers containing membrane inserts of 8 μm pore size and invasion chambers with same membrane coated with collagen were purchased from Millipore as assay kit. CD44 antibody was purchased from Epitomics. p53 antibody was obtained from Santa Cruz Biotechnology. Actin and tubulin antibodies, TRIzol RNA isolation kit, and mevalonolactone were purchased from Sigma. The primers to detect CD44 mRNA (forward, 5′-CCACGTGGAGAAAAATGGTC-3′; reverse, 5′-CATTGGGCAGGTCTGTGAC-3′) and p53 mRNA (forward, TGGTAATCTACTGGGACGGA; reverse, GCTTAGTGCTCCCTGGGGGC) and CD44 promoter spanning p53-binding element (forward, 5′-TTTACGGTTCGGTCATCCTC; reverse, 5-TGCTCTGCTGAGGCTGTAAA) were synthesized in the core facility at the University of Texas Health Science Center. The GAPDH and PUMA[5] primer sets (including forward and reverse primers) and SYBR Green real time PCR master mix were obtained from SuperArray Biosciences. Chromatin immunoprecipitation kit was purchased from Active Motif. shCD44–2 pRRL, shp53 pLKO.1 puro, and CD44-Luc vectors were purchased from Addgene, and these plasmids were submitted by Dr. Robert Weinberg (Whitehead Institute) (19). p53-Luc reporter plasmid containing consensus p53 binding element (TGCCTGGACTTTGCCTGG) driving the luciferase gene (20) was obtained from Stratagene.

Preparation of Mevalonate—The mevalonate was prepared as suggested by the vendor. Briefly, 40 mg of mevalonolactone was dissolved in 500 μl of water followed by addition of 500 μl of 0.5 M KOH. Finally, the pH was adjusted to 7.5 with 0.1 M HCl. The cells were incubated with 250 μM mevalonate for 6 h prior to treatment with statins for the indicated periods of time.

Animal Study to Generate Mammary Tumor and Osteolytic Metastasis of Breast Cancer Cells—Immunocompromised nude mice were purchased from the National Institutes of Health Animal Facility. All animal protocols were approved by the Institutional Animal Care and Use Committee. The animals were maintained on normal laboratory chow. Mice were injected with 5 mg/kg body weight of simvastatin every day for 7 days. Control mice received phosphate-buffered saline. Mice were injected with 10^5 MDA-MB-231 human breast cancer cells in the left cardiac ventricle, as described previously, and maintained on same dose of simvastatin (21). Four weeks after injection of the breast cancer cells, deeply anesthetized mice were x-rayed using a Faxitron radiographic inspection unit as described previously (21). The radiolucent areas of the hind limb on the x-ray plate were marked. The areas were quantified using a computer-assisted BIOQUANT image analysis program.

To generate mammary tumor, 10^6 MDA-MB-231 cells were injected into the mammary fat pad of the control and simvastatin-treated mice. The mice were sacrificed at 3 weeks, and tumors were excised and frozen and lysates prepared as described previously (10).

Scratch Assay—MDA-MB-231 and BT-20 cells were grown in monolayer in the presence and absence of 5 μM simvastatin or lovastatin. A scratch was engineered manually in the center of the monolayer with a pipette tip (22). The cell monolayer was incubated for the indicated period of time to allow cell migration into the scratched area. The monolayer was photomicrographed. The distance between the two cell edges was quantified using the BIOQUANT software (23).

Migration and Invasion Assay—The MDA-MB-231 and BT-20 breast cancer cells were incubated with 5 μM simvastatin or lovastatin prior to trypsinization. 6×10^4 cells were seeded in trans-well chambers containing membrane with 8 μm pore size. The migration chambers were placed on 24-well plates for incubation at 37 °C for 6 h. The cells on top of the chamber were removed with a cotton swab. The cells that migrated to the bottom of the membrane were stained with the reagent pro-

[5] The abbreviation used is: PUMA, p53 up-regulated modifier of apoptosis.

vided in the kit. The stain was extracted using the extraction buffer according to the vendor's instructions. The absorbance of the eluted stain was determined at 590 nm. The absorbance is directly proportional to the number of cells that migrated. The absorbance was taken as arbitrary units to represent the number of cells that migrated. For invasion assay, the cells were seeded in trans-well chambers with 8 μm of membrane coated with collagen. The rest of the assays were performed as described above. The migrated (invaded) stained cells on the other side of the membrane were photographed and counted before extraction of the stain. The absorbance of the eluted stain was plotted as direct measure of invaded cells.

Preparation of Cell and Tumor Lysates—Excised tumors were homogenized as described previously (10, 24). Briefly, the tumors were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.05% aprotinin, and 1% Nonidet P-40). The homogenates were centrifuged at 12,000 $\times$ *g* at 4 °C for 30 min. The cleared supernatant was used to determine the protein concentration using Bio-Rad reagent. To prepare MDA-MB-231 and BT-20 cell lysates, the treated or transfected cells were lysed in RIPA at 4 °C for 20 min and centrifuged as described above. The supernatant was collected and protein concentration determined as before.

Immunoblotting—Equal amounts of tumor or cell lysates were separated by SDS-PAGE. The separated proteins were electrotransferred to the PVDF membrane. The membrane containing the proteins was used for immunoblotting with required antibodies essentially as described previously (25–27). The protein bands were scanned and quantified as a ratio to loading control. The histograms are presented for quantification of the data.

RNA Extraction and Real Time RT-PCR—Total RNAs were prepared from breast cancer cells using TRIzol RNA extraction kit as described previously (23). Total RNA was reverse-transcribed and qRT-PCR carried out using SYBR Green master mix and primers specific for CD44, p53, and PUMA. The PCR amplification was performed in 7900HT sequence detection system from Applied Biosystems. The amplification conditions are as follows: CD44 and PUMA, 94 °C for 10 min followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; p53, 94 °C for 10 min followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Relative mRNA expression was calculated using $\Delta\Delta C_t$ method.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed using a kit as described previously (28). Briefly, MDA-MB-231 breast cancer cells were treated with simvastatin followed by preparation of sheared chromatin. Sheared chromatins were cleared and incubated with protein G-agarose beads and used as the input control. Sheared chromatins were also incubated with IgG or anti-p53 antibody to immunoprecipitate the p53-bound DNA fragment. The eluted DNA was PCR-amplified with CD44 promoter-specific primers and analyzed by agarose gel electrophoresis. Also, the amplification reaction was performed using a real time PCR machine as described above. PCR condition was as follows: 94 °C for 10 min followed by 37 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s.

Transfection and Luciferase Activity—MDA-MB-231 mammary cancer cells were transfected with the indicated expression vectors and luciferase reporter constructs using FuGENE HD as described previously (24, 26, 27). Cell lysates were used for immunoblotting or luciferase assay as described previously using the luciferase assay kit (24, 26, 27). The data were corrected against protein content. Mean $\pm$ S.E. of triplicate measurements is shown.

Statistical Analysis of the Data—The significance of the data was determined using analysis of variance followed by paired *t* test or Student-Newman-Keuls analysis as described previously (10, 23, 26, 27). The changes were considered significant if the *p* value was less than 0.05.

RESULTS

Simvastatin Attenuates Breast Cancer Cell Metastasis to Bone—To test the efficacy of simvastatin on metastasis of breast cancer cells, we chose MDA-MB-231 human mammary tumor cells, which have been shown to metastasize to bone to form osteolytic lesions (21). Saline- or simvastatin-treated nude mice were injected with MDA-MB-231 breast cancer cells. As expected and shown in the x-rays, breast cancer cells metastasized to bone to produce significant osteolytic lesions in the hind limbs of nude mice (Fig. 1*A*, *upper panels*, indicated by *arrows*). However, in a simvastatin-treated group, bone lesions were markedly reduced (Fig. 1*A*, *bottom panels*). Quantification of the osteolytic areas showed significant prevention of breast cancer metastasis to bone in the simvastatin-treated animals (Fig. 1*B*). These results demonstrate that simvastatin prevents breast cancer metastasis to bone.

Next, we examined the effect of simvastatin on migration of breast cancer cells *in vitro*. A scratch assay using MDA-MB-231 cell monolayer was used. In the presence of serum, the cells showed significant migration between 12 and 24 h (Fig. 2*A*, *control*). Simvastatin markedly inhibited the migration of breast cancer cells (Fig. 2, *A* and *B*). Similar results were observed when the cells were incubated in the absence of serum (Fig. 2, *C* and *D*). Another member of the statin family of drugs, lovastatin, also decreased the migration of MDA-MB-231 cells in the presence and absence of serum (supplemental Fig. S1, *A–D*). To test the action of statins, we used another human breast cancer cell, BT-20, which has been shown to metastasize to organs, including pelvis, liver, and kidney (29). Both simvastatin and lovastatin significantly blocked migration of these cells (supplemental Fig. S2, *A* and *B*). To confirm this observation, we performed a trans-well assay to score migration of cells. Incubation of MDA-MB-231 cells with simvastatin significantly attenuated their migration (Fig. 2*E*).

Actual metastasis process is initiated by local invasion of cancer cells (intravasation). To examine the effect of simvastatin on the metastatic potential of MDA-MB-231 cells *in vitro*, we carried out an invasion assay using collagen-coated membrane in trans-wells. Untreated MDA-MB-231 cells showed marked invasion (Figs. 2*F*, *control*). Simvastatin significantly inhibited the invasion of tumor cells (Fig. 2, *F–H*). Similarly, lovastatin markedly blocked the invasion of MDA-MB-231 cells (supplemental Fig. S3, *A* and *B*). Furthermore, both simvastatin and lovastatin attenuated the invasion of BT-20 human breast can-

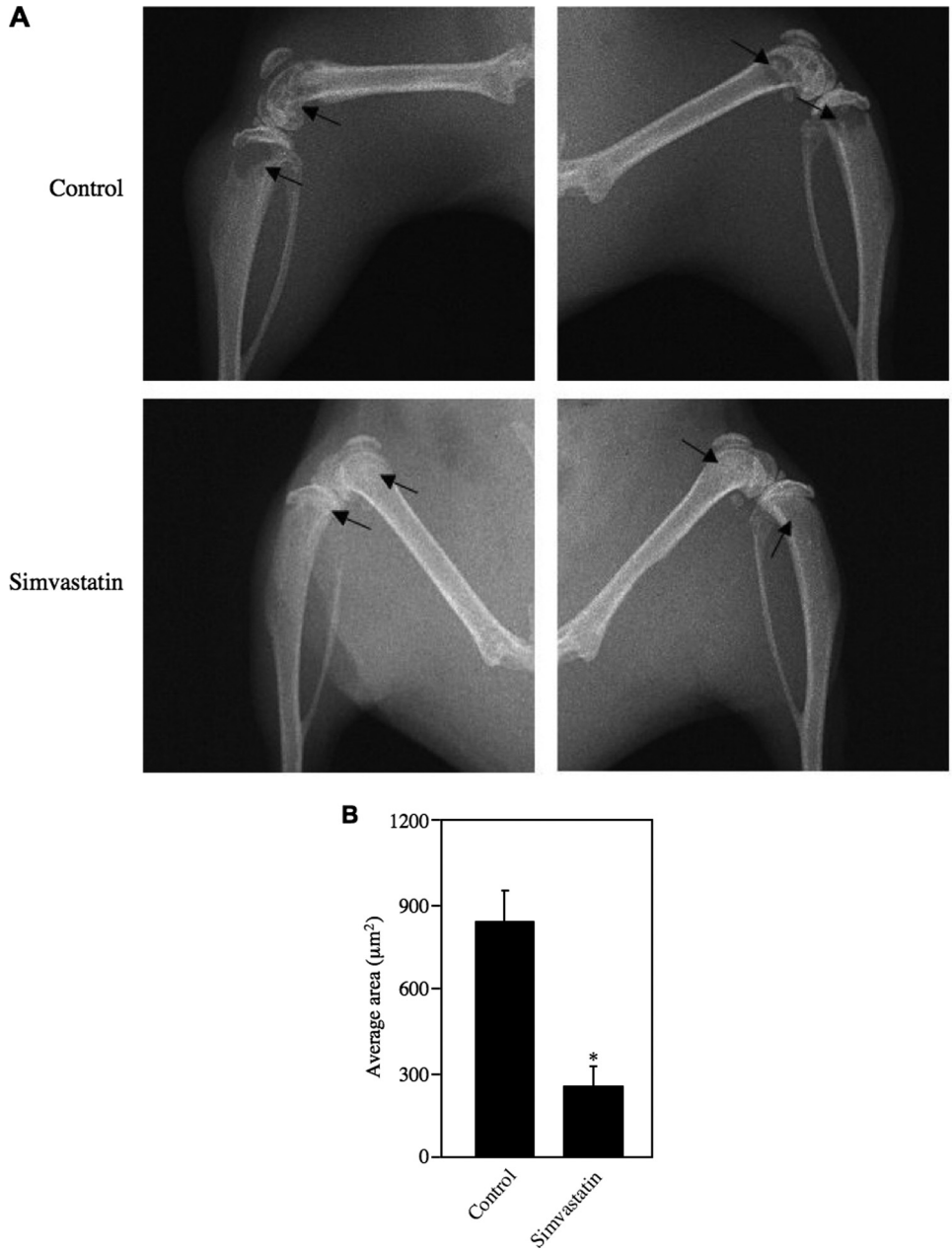
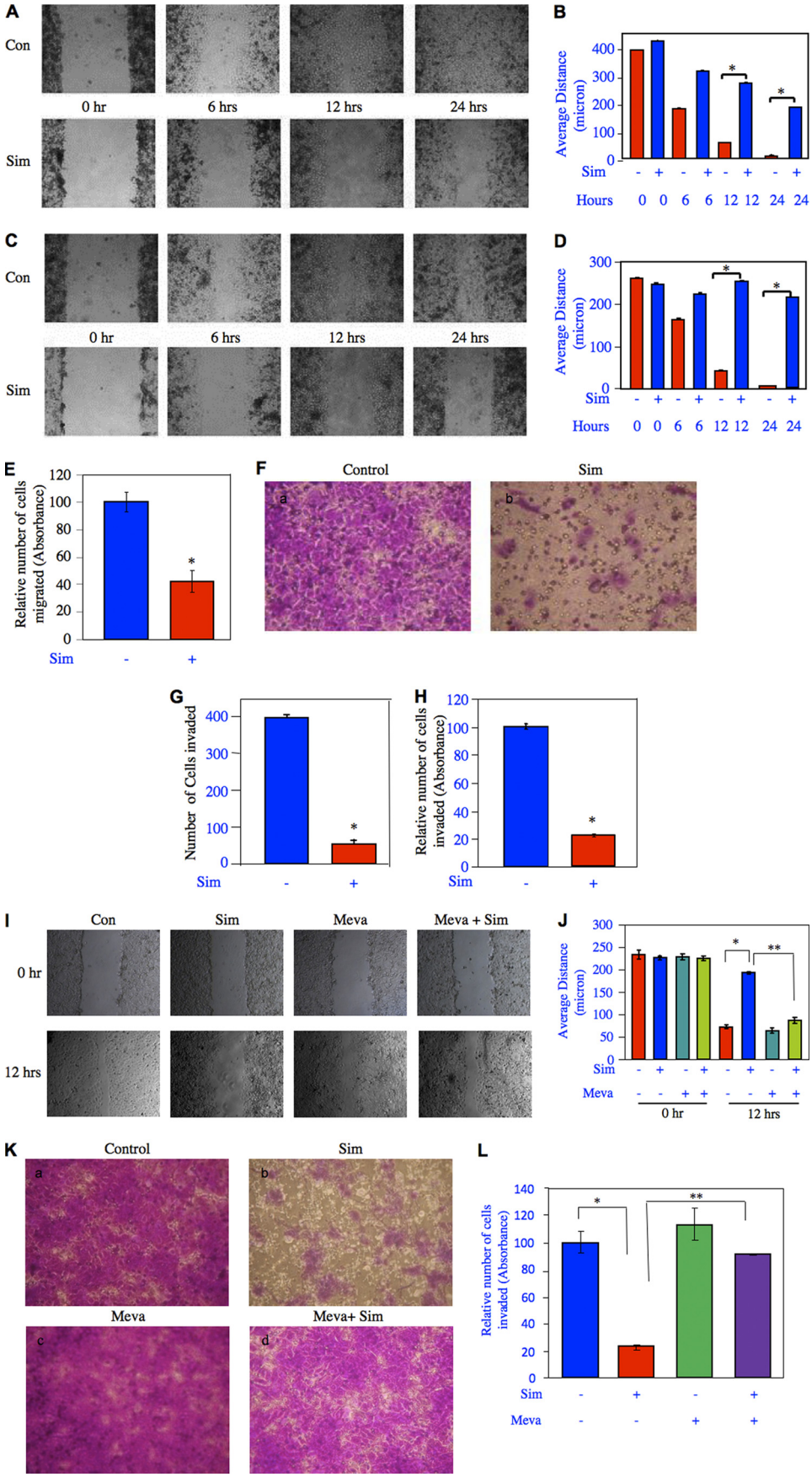


FIGURE 1. **Effect of simvastatin on breast cancer metastasis to bone.** *A,* inhibition of osteolytic lesion formation in simvastatin-treated mice. Two groups of mice treated with vehicle and simvastatin, respectively, received MDA-MB-231 human breast cancer cells in their left cardiac ventricle, and the osteolytic lesions were visualized by x-rays as described under "Experimental Procedures" (21). Hind limb x-rays from two control mice (*top panels*) and two simvastatin-treated animals (*bottom panels*) are shown. *Arrows* indicate the osteolytic lesions in the joints. *B,* quantification of the osteolytic lesions in *A*. The lesions areas in the control and simvastatin-treated mice were marked and quantified as described under "Experimental Procedures." $n = 6$; *, $p = 0.001$ *versus* control.

cer cells (supplemental Fig. S2, *C* and *D*). These data suggest that statins may attenuate breast cancer cell metastasis to different organs.

Statins inhibit the rate-limiting enzyme HMG-CoA reductase in the mevalonate pathway (1). To test the involvement of HMG-CoA reductase in the breast cancer cell migration, we incubated MDA-MB-231 cells with mevalonate prior to treatment with simvastatin. The results showed that mevalonate significantly prevented the inhibition of migration induced by simvastatin (Fig. 2, *I* and *J*). Similar results were obtained with BT-20 human breast tumor cells (supplemental Fig. S4, *A* and *B*). Mevalonate also blocked the inhibitory action of simvastatin on MDA-MB-231 breast cancer cell invasion (Fig. 2, *K* and *L*). These results suggest that statins may use mevalonate pathway to prevent breast cancer cell metastasis.

Statins Down-regulate CD44 to Suppress Breast Cancer Cell Migration—To investigate the mechanism of statin-mediated inhibition of breast cancer cell invasion, we considered the involvement of CD44, as this cell surface antigen is enriched in epithelial tumor-initiating and metastatic cancer cells (19, 30–36). Furthermore, a role for CD44 in cancer metastasis has been established (30, 31, 35). Incubation of MDA-MB-231 cells with simvastatin significantly reduced the levels of CD44 protein (Fig. 3*A*, *left panel*). Similarly, lovastatin inhibited the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expression of CD44 protein (Fig. 3*A*, *right panel*). Mevalonate prevented the inhibition of CD44 protein expression by simvastatin (supplemental Fig. S5) Real time RT-PCR revealed that both simvastatin and lovastatin significantly decreased CD44 mRNA (Fig. 3*B*). Similar results were obtained with BT-20 human breast cancer cells (supplemental Fig. S6, *A* and *B*). To examine the involvement of a transcriptional mechanism for the expression of CD44, we used a reporter vector (CD44-Luc) in which the CD44 promoter drives the luciferase gene. Transient transfection assays were performed in MDA-MB-231 and BT-20 cells using this plasmid. Both simvastatin and lovastatin significantly inhibited the reporter activity (Fig. 3*C* and supplemental Fig. S6*C*), indicating that statins regulate the expression of CD44 by a transcriptional mechanism.

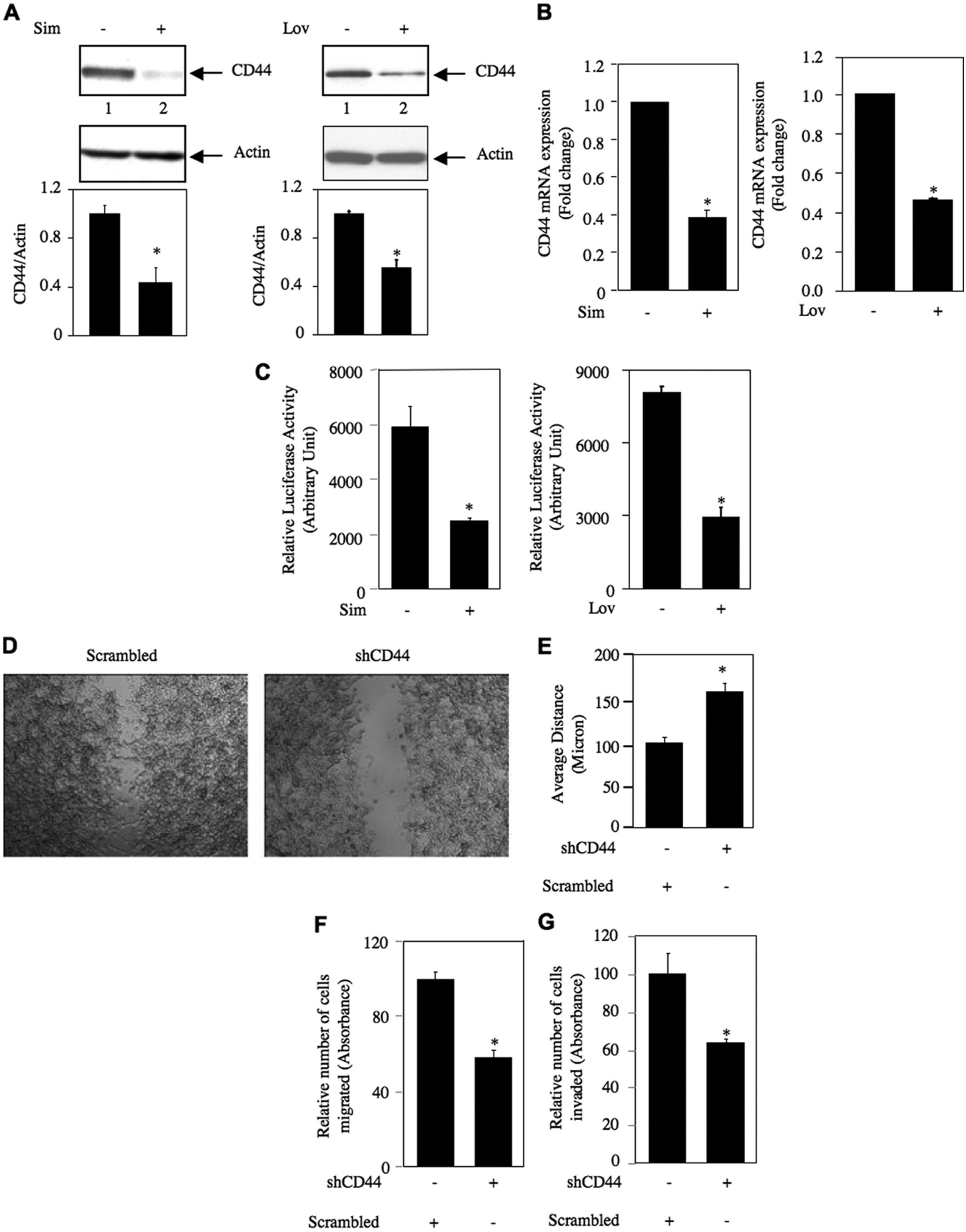
Because statins suppressed both migration and invasion of MDA-MB-231 cells and expression of CD44, we examined the direct involvement of CD44 in migration using shRNA-directed down-regulation of this protein. Transfection of shCD44, which inhibits the expression of CD44 protein (supplemental Fig. S7), significantly prevented the migration of MDA-MB-231 cells in a scratch assay (Fig. 3, *D* and *E*). Similarly, transfection of shCD44 inhibited the migration of MDA-MB-231 cells as judged by trans-well assay (Fig. 3*F*). Furthermore, shCD44 significantly decreased the invasion of MDA-MB-231 mammary carcinoma cells (Fig. 3*G*). Similarly, shCD44 markedly blocked invasion of the BT-20 breast cancer cells (supplemental Fig. S8, *A–C*). These results indicate a mechanism involving CD44 in statin-mediated attenuation of breast cancer cell migration and invasion.

Statins Target p53 to Repress CD44 Expression—The transcriptional repression of CD44 by statins (Fig. 3*C*) prompted us to search for a transcription factor that would mediate the effect of the statins. A correlation describing increased CD44 expression and p53 negativity in mammary cancer cells has recently been reported (19, 37). Therefore, we examined whether statins target p53. Incubation of MDA-MB-231 cells with simvastatin as well as with lovastatin significantly increased p53 mRNA and protein levels (Fig. 4, *A* and *B*). Both simvastatin and lovastatin also increased the expression of p53 mRNA and protein in BT-20 breast cancer cells (supplemental Fig. S9, *A* and *B*). The increase in protein abundance was associated with phosphorylation of p53 (supplemental Fig. S10), indicating that statins may regulate transcriptional activity of p53. In fact, both simvastatin and lovastatin robustly promoted the transcription of a p53-responsive reporter gene containing the p53 consensus sequence (Fig. 4*C*). Similar results were obtained in the BT-20 human breast tumor cells (supplemental Fig. S9*C*). To directly test the biological consequence of simvastatin-induced p53 up-regulation, we tested the expression of a p53 target gene, PUMA (38). Incubation of MDA-MB-231 cells with simvastatin significantly augmented the expression of PUMA mRNA (supplemental Fig. S11). These results suggest that statins may utilize p53 to regulate CD44 expression in breast tumor cells.

We have shown above that statins repress CD44 expression by a transcriptional mechanism (Fig. 3, *B* and *C*). Recently, Godar *et al.* (19) has identified the noncanonical p53-binding site in the CD44 promoter. In the MDA-MB-231 breast cancer cells, we tested whether endogenous p53 occupies this site in the CD44 promoter by performing ChIP assay (28). As shown in Fig. 4*D*, we detected physical association of p53 with the CD44 promoter sequence in MDA-MB-231 cells. Next, we examined the effect of simvastatin on binding of endogenous p53 to the CD44 promoter. Fig. 4*E* shows significant increase in interaction of p53 with the CD44 promoter in response to simvastatin. These results conclusively demonstrate that simvastatin-induced elevation of p53 expression results in marked increase in p53 recruitment to the CD44 promoter. Furthermore, these data indicate that simvastatin-mediated increase in p53 binding to the CD44 promoter may suppress its transcription and protein expression.

To address further the potentially important role of p53 in regulation of CD44 expression, we used shRNA-directed knockdown of p53. Inhibition of p53 expression in MDA-MB-231 cells by transfection of shp53 plasmid significantly increased the levels of CD44 protein (Fig. 5*A*). Similar to this observation, transfection of shp53 promoted the expression of CD44 mRNA (Fig. 5*B*). Similar results were obtained when shp53 was transfected into the BT-20 breast cancer cells (supplemental Fig. S12, *A* and *B*). These results indicate that p53 acts as a transcriptional repressor for CD44 in these breast cancer cells. To directly examine the transcriptional role of p53 on CD44 expression, we transfected the MDA-MB-231 and BT-20 cells with the reporter plasmid CD44-Luc. Transfection of shp53 significantly increased the transcription of CD44 (Fig. 5*C* and supplemental Fig. S12*C*). Furthermore, to confirm the transcriptional role of p53, we tested the expression of PUMA mRNA whose expression is up-regulated by p53. shp53 signif-

FIGURE 2. **Simvastatin inhibits breast cancer cell migration and invasion via mevalonate pathway.** *A–D,* simvastatin inhibits migration of breast cancer cells. MDA-MB-231 breast carcinoma cells grown in 10% serum-containing medium (*A*) or in serum-free medium (*C*) were scratched and incubated with 5 μM simvastatin (*Sim*) for the indicated times. The cell monolayer was photomicrographed. The distance between the cell edges was determined by BIOQUANT as described under "Experimental Procedures." *Con*, control. *B* and *D* represent the quantification of *A* and *C*, respectively. Mean ± S.E. of five independent field measurements is shown. *, $p < 0.005$ *versus* timed control. *E,* simvastatin blocks migration of breast cancer cells. MDA-MB-231 human breast tumor cells were incubated with 5 μM simvastatin for 24 h. The cells were then transferred to trans-wells in the same medium on membrane with 8-μm pore size as described under "Experimental Procedures." The migrated cells on the other side of the membrane were stained. The absorbance of the eluted stain was measured at 590 nm. The absorbance is proportional to the number of cells migrated. Mean ± S.E. of triplicate wells is shown. *, $p = 0.04$ *versus* control. *F–H,* simvastatin inhibits breast cancer cell invasion. MDA-MB-231 cells were incubated with 5 μM simvastatin for 24 h and placed in a trans-well chamber with collagen-coated membrane (8-μm pore size) as described under "Experimental Procedures." The cells that invaded to the other side of the membrane were stained and photographed (*F*). Number of stained cells was counted (*G*). Also, the stains were eluted, and the absorbance was measured at 590 nm (*H*). *G*, mean ± S.E. of 6 independent fields from triplicate wells is shown *, $p = 0.003$ *versus* control. *H*, mean ± S.E. of four measurements is shown.; *, $p = 0.001$ *versus* control. *I–L,* mevalonate prevents simvastatin-induced inhibition of migration and invasion. MDA-MB-231 cells were treated with 250 μM mevalonate (*Meva*) for 6 h prior to incubation with 5 μM simvastatin and used for scratch assay (*I*) and for invasion assay (*K*) as described in the *A* and *F*, respectively. *J,* quantification of the results presented in *I*. *, $p < 0.001$ *versus* control at 12 h; **, $p < 0.001$ *versus* simvastatin-treated at 12 h. *L,* quantification of the results presented in *K*. The stains were eluted from the cells at the bottom of the membrane in *K,* and the absorbance was determined at 590 nm. Mean ± S.E. of triplicate measurements is shown. *, $p < 0.001$ *versus* control; **, $p < 0.001$ *versus* mevalonate-treated.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Relative number of cells migrated (Absorbance)
120
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shCD44 - +
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G
Relative number of cells invaded (Absorbance)
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60
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shCD44 - +
Scrambled + -

icantly inhibited PUMA mRNA expression in MDA-MB-231 cells (Fig. 5*D*). It should be noted that despite the presence of mutated p53R280K in MDA-MB-231 cells (39), it showed appreciable transcriptional activity (Figs. 4*C* and 5, *B–D,* and supplemental Fig. S11). To rule out the possibility of contamination of our MDA-MB-231 cells with cells containing wild type p53, we cloned and sequenced part of the expressed p53 mRNA spanning the codon R280. Results from two independent pairs of primers showed the presence of mutation, as originally reported (supplemental Fig. S13) (39).

p53 Regulates Migration of Human Breast Cancer Cells—We have shown above that p53R280K retains transcriptional function to regulate expression of CD44 in MDA-MB-231 breast cancer cells (Fig. 5, *A–C*). We examined the involvement of endogenous p53R280K in migration of these cells. Transfection of shp53 into MDA-MB-231 cells increased migration of these cells as examined by scratch assay (Fig. 6, *A* and *B*). Trans-well assay showed pronounced migration in shp53-transfected cells (Fig. 6*C*). Furthermore, down-regulation of endogenous p53R280K increased invasion of MDA-MB-231 cells (Fig. 6*D*). Similarly, sh53-mediated reduction in p53 expression in BT-20 human breast cancer cells (supplemental Fig. S14*A*) significantly increased the invasion (supplemental Fig. S14, *B* and *C*). These results demonstrate that the mutant p53R280K in the MDA-MB-231 as well as the p53 protein present in BT-20 cells have sufficient biological activity.

Simvastatin Regulates p53 and CD44 Expression in the MDA-MB-231-generated Tumors—We have shown above that simvastatin increases p53 expression in MDA-MB-231 breast cancer cells (Fig. 4, *A–C*). We investigated its expression *in vivo*. We used MDA-MB-231-induced tumors in nude mice. Immunoblotting of the tumor lysates from the control and simvastatin-treated mice was performed using p53 antibody. It is interesting that expression of p53 in the tumor xenografts is significantly low (Fig. 7*A*, *lanes 1–3*). Levels of p53 were significantly elevated in the extracts of tumors isolated from animals treated with simvastatin (Fig. 7, *A*, compare *lanes 4–6* with *lanes 1–3* and *B*). In contrast to this observation, expression of CD44 in the tumor samples prepared from control mice was high (Fig. 7*C*, *lanes 1–3*). However, simvastatin-treated mice exhibited significantly reduced levels of CD44 (Fig. 7, *C*, compare *lanes 4–6* with *lanes 1–3* and *D*). These results for the first time indicate that simvastatin induces a reciprocal regulatory mechanism for expression of p53 and CD44 in breast tumors of mice.

DISCUSSION

The osteolytic nature of breast cancer metastasis to bone results from complex interaction between breast tumor cells and surrounding bone marrow stroma, which leads to bone destruction and intense pain. In this study, we demonstrate a novel action of simvastatin in preventing the osteolysis resulting from human breast cancer cell metastasis. We show that simvastatin inhibits the metastatic MDA-MB-231 cell migration and invasion *in vitro*. Our results provide evidence that simvastatin attenuates the expression of CD44, which regulates migration and invasion of breast cancer cells. We show that simvastatin increases the levels of mutated p53 in MDA-MB-231 cells to repress the expression of CD44. Finally, we demonstrate an inverse correlation between expression of p53 and CD44 in the simvastatin-treated mice xenografts.

Expression of functionally distinct class of genes endows the tumor cells to initiate metastasis and determines its progression and virulence. Cellular motility and degradation of basement membrane mediate cancer cell dissemination into the circulation (40). On the other hand, modification of cytoskeletal components, such as RhoC may contribute to metastatic dissemination (41). RhoA and RhoC, which are modified by geranylgeranyl moiety produced by the HMG-CoA reductase-mediated mevalonate pathway, have been shown to be up-regulated in many cancers, including breast cancer, and induce epithelial-mesenchymal trans-differentiation necessary for metastasis (2). Our observation that simvastatin blocks breast cancer metastasis to bone (Fig. 1) and that both simvastatin and lovastatin inhibit *in vitro* migration and invasion of MDA-MB-231 cells may result from inhibition of geranylgeranylation of these Rho proteins. In fact, our results show the involvement of HMG-CoA reductase in the invasion of breast cancer cells (Fig. 2, *I–L*).

What induces the breast cancer cells to metastasize to distant sites in an organ-specific manner is not clear. However, emerging results shed light on lung-specific metastasis of human MDA-MB-231 breast cancer cells, indicating that expression of specific gene sets may be involved in this phenomenon. For example, epiregulin, MMP1, and Cox2 are expressed mainly in the solid breast tumor and promote angiogenesis. However, when they are manifested in the disseminated cancer cells in the circulation, they increase the extravasation of these cells into lung parenchyma (14). Expression of angiopoietin-like 4 in the disseminated breast cancer cells also contributes to the pul-

FIGURE 3. **Statins inhibit expression of CD44 to suppress migration and invasion of breast cancer cells.** *A* and *B,* statins block CD44 expression. MDA-MB-231 breast carcinoma cells were incubated with 5 μM simvastatin (*Sim*) (*left panel*) or lovastatin (*Lov*) (*right panel*) for 24 h. *A,* equal amounts of cell lysates were immunoblotted with CD44 and actin antibodies, respectively. The *bottom panels* show quantification of the protein bands. $n = 3$; *, $p = 0.005$ *versus* control for the *left panel*; *, $p = 0.01$ *versus* control for the *right panel*. *B,* total RNA from these cells was used to detect CD44 mRNA by real time RT-PCR as described under "Experimental Procedures." Mean ± S.E. of triplicate measurements is shown. *, $p = 0.002$ *versus* control for the *left panel*; *, $p = 0.003$ *versus* control for the *right panel*. *C,* statins inhibit transcription of CD44. MDA-MB-231 cells were transfected with CD44-Luc reporter plasmid prior to incubation with 5 μM simvastatin (*left panel*) or lovastatin (*panel*). The cell lysates were assayed for luciferase activity as described under "Experimental Procedures." Mean ± S.E. of triplicate measurements is shown. *, $p = 0.04$ *versus* control for the *left panel*; *, $p = 0.006$ *versus* control for the *right panel*. *D–G,* expression of shCD44 blocks migration and invasion of the breast cancer cells. *D*, MDA-MB-231 cells were transfected with shCD44 or scrambled vector plasmid followed by applying a scratch. The photograph was taken after 24 h. *E* shows quantification of the data in *D*. The distance between the cell edges was determined by BIOQUANT as described under "Experimental Procedures." Mean ± S.E. of four independent fields is shown. *, $p = 0.02$ *versus* scramble-transfected. *F*, shCD44-transfected MDA-MB-231 cells were used in a trans-well assay as described in the Fig. 2*E*. Mean ± S.E. of four measurements is shown. *, $p = 0.002$ *versus* scrambled vector-transfected. *G*, shCD44-transfected MDA-MB-231 cells were used to perform invasion assay using trans-well chambers with collagen-coated membrane as described in Fig. 2*H*. The invaded cells on the bottom of the membrane were stained, and the stain was extracted to determine the absorbance at 590 nm. The absorbance is proportional to the number of cells invaded. Mean ± S.E. of four measurements is shown. *, $p = 0.001$ *versus* scrambled vector-transfected.

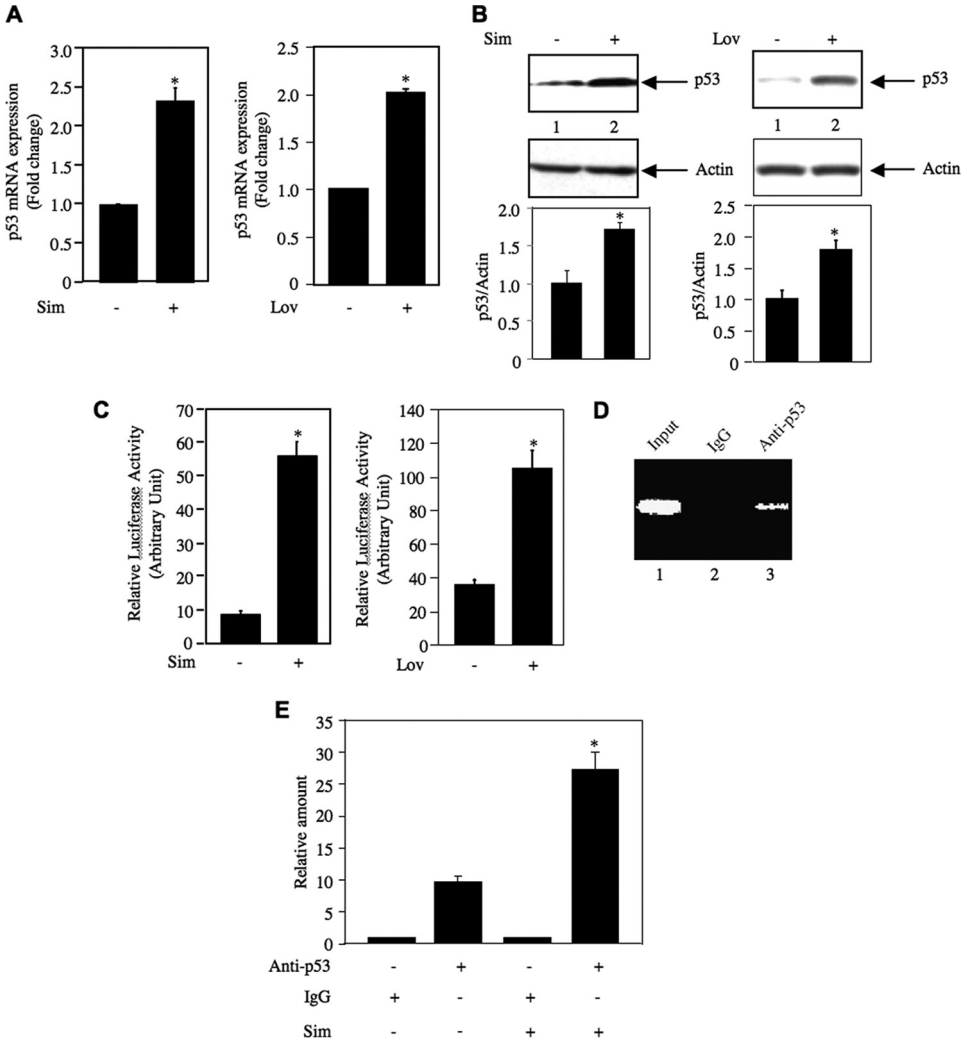

FIGURE 4. **Statins increase p53 in breast cancer cells.** *A* and *B,* statins promote p53 mRNA and protein levels. MDA-MB-231 cells were incubated with 5 μM simvastatin (*Sim*) (*left panel*) or lovastatin (*Lov*) (*right panel*). Total RNA was used to detect p53 mRNA by real time RT-PCR as described under "Experimental Procedures" (*A*). Mean ± S.E. of triplicate measurements is shown. *, $p = 0.016$ *versus* control for the *left panel*; *, $p = 0.001$ *versus* control for the *right panel*. *B*, cell lysates were immunoblotted with p53 and actin antibodies, respectively. The *bottom panel* shows quantification of the protein bands. $n = 3$; *, $p = 0.01$ *versus* control for the *left panel*; *, $p = 0.006$ *versus* control for the *right panel*. *C,* statins increase p53-dependent reporter transcription. p53-Luc reporter construct was transfected into MDA-MB-231 cells followed by incubation with 5 μM simvastatin (*left panel*) or lovastatin (*right panel*). The cell lysates were used for luciferase assay are as described under "Experimental Procedures." Mean ± S.E. of triplicate measurements is shown. *, $p = 0.008$ *versus* control for the *left panel*; *, $p = 0.003$ *versus* control for the *right panel*. *D,* ChIP assay to determine binding of p53 to CD44 promoter. Fragmented chromatins from MDA-MB-231 cells were incubated with IgG or anti-p53 antibody as described under "Experimental Procedures." The bound DNA was eluted and amplified with the CD44 promoter-specific primers flanking the p53 binding element as described under "Experimental Procedures." The amplified products were separated by agarose gel electrophoresis. *E,* simvastatin increases binding of p53 to CD44 promoter. Chromatins were prepared from MDA-MB-231 cells incubated with 5 μM simvastatin. Fragmented chromatin preparations were used for ChIP assay as described in *D* except the amplification was performed using SYBR Green real time PCR as described under "Experimental Procedures." Relative amount of bound p53 was calculated by the ratio of ChIPed DNA to input control DNA. Mean ± S.E. of triplicate measurements is shown. *, $p < 0.001$ *versus* anti-p53 alone without simvastatin.

monary metastasis by dissociation of endothelial junctions (15). Although a gene expression profile indicated involvement of fibroblast growth factor signaling for breast cancer relapse in bone, a tight association between primary solid tumor gene expression and bone metastasis has not been reported (42, 43). However, the fenestrated endothelia of the bone marrow capillaries, which facilitate trafficking of hematopoietic cells, allow free flow of breast cancer cells into bone marrow, and thus bypass the requirement of expression of specific genes for extravasation (43). The bone marrow serves as a permissive niche for residence of disseminated breast cancer cells. In fact, the mesenchymal cells in the bone marrow produce the stromally derived factor-1 (SDF1) that may bind to its receptor C*X*C chemokine receptor-4 (CXCR4) present on the surface of disseminated breast cancer cells to maintain prolonged survival (43). The colonized breast cancer cells secrete osteoclastogenic factors, namely IL-6, TNF-α, IL-11, GM-CSF, and parathyroid hormone-related peptide (18, 44–46). Many of these factors act on osteoclasts to induce production and secretion of receptor activator of NFκB ligand that promote osteoclastogenesis and cause the formation of osteolytic lesions in bone. Our results demonstrate that the HMG-CoA reductase inhibitor simvastatin significantly prevents the formation of osteolytic lesions caused by the metastasis of human MDA-MB-231 breast cancer cells to the bone (Fig. 1). This effect of simvastatin may result from its action on the colonized breast cancer cells in

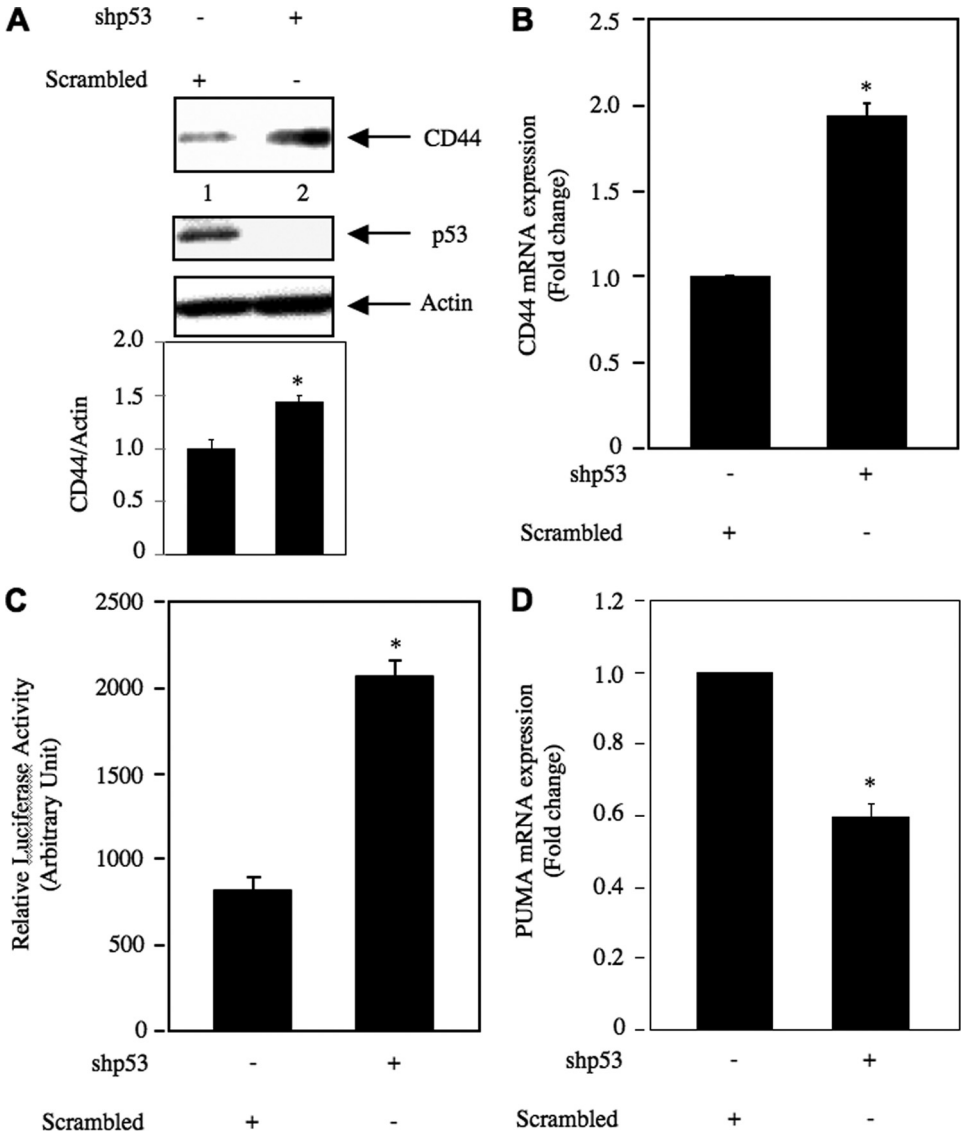

FIGURE 5. **Down-regulation of p53 increases expression of CD44 in human breast cancer cells.** *A* and *B,* MDA-MB-231 cells were transfected with shp53 or scramble plasmid. *A,* cell lysates were immunoblotted with CD44, p53, and actin antibodies, respectively. The *bottom panel* shows quantification of CD44 protein bands. $n = 3$; *, $p = 0.02$ *versus* scramble-transfected. *B,* total RNA was used to detect CD44 mRNA using real time RT-PCR as described under "Experimental Procedures." Mean ± S.E. of triplicate measurements is shown. *, $p = 0.001$ *versus* scramble-transfected. *C,* down-regulation of p53 increases CD44 transcription. MDA-MB-231 cells were transfected with CD44-Luc along with shp53 or scramble vector plasmid. The cell lysates were used for luciferase activity as described under "Experimental Procedures." Mean ± S.E. of triplicate measurements is shown. *, $p = 0.01$ *versus* scramble-transfected. *D,* inhibition of p53 blocks PUMA expression. MDA-MB-231 cells were transfected with shp53 or scramble vector. Total RNA was used to detect PUMA mRNA as described under "Experimental Procedures." Mean ± S.E. of triplicate measurements is shown. *, $p = 0.008$ *versus* scramble-transfected.

bone to inhibit the production and secretion of the osteoclastogenic factors described above. However, our observations *in vitro* show that statins markedly inhibit the migration as well as invasion of the MDA-MB-231 mammary tumor cells (Fig. 2 and supplemental Figs. S1 and S3). Furthermore, we demonstrate that statins block the migration and invasion of another metastatic human breast cancer cell BT-20 (supplemental Fig. S2). These results indicate that statins may attenuate the metastasis of breast cancer cells.

Gene expression profiling classified the breast cancer cells into five different classes: basal, luminal, mesenchymal, myoepithelial, and Erb2-positive (47, 48). Patients with basal type mammary tumor have worst prognosis (11). According to the gene expression profile, basal and mesenchymal breast cancer cells share similar features. Basal/mesenchymal and myoepithelial cells contain high levels of CD44 transmembrane receptor, which exhibits a lineage for the presence of breast cancer stem cells and cells that have undergone epithelial-mesenchymal trans-differentiation (32, 49). Although these cells represent a small proportion of cells in solid tumor, only these cells when injected into nude mice formed tumor (32). These cells also contribute to migration and cancer metastasis (50). However, using $p53^{+/tm1}CD44^{-/-}$ mice, Weber *et al.* (31) showed no effect of CD44 on tumor incidence and survival of osteosarcomas. Rather absence of CD44 significantly prevented their dissemination to form micro- and macro-metastasis in lung and liver. In fact, the invasive property of the human breast cancer cells was tightly linked to the presence of CD44 (51). In MDA-MB-231 breast cancer cells, which represent the mesenchymal phenotype, 85% of the population was positive for

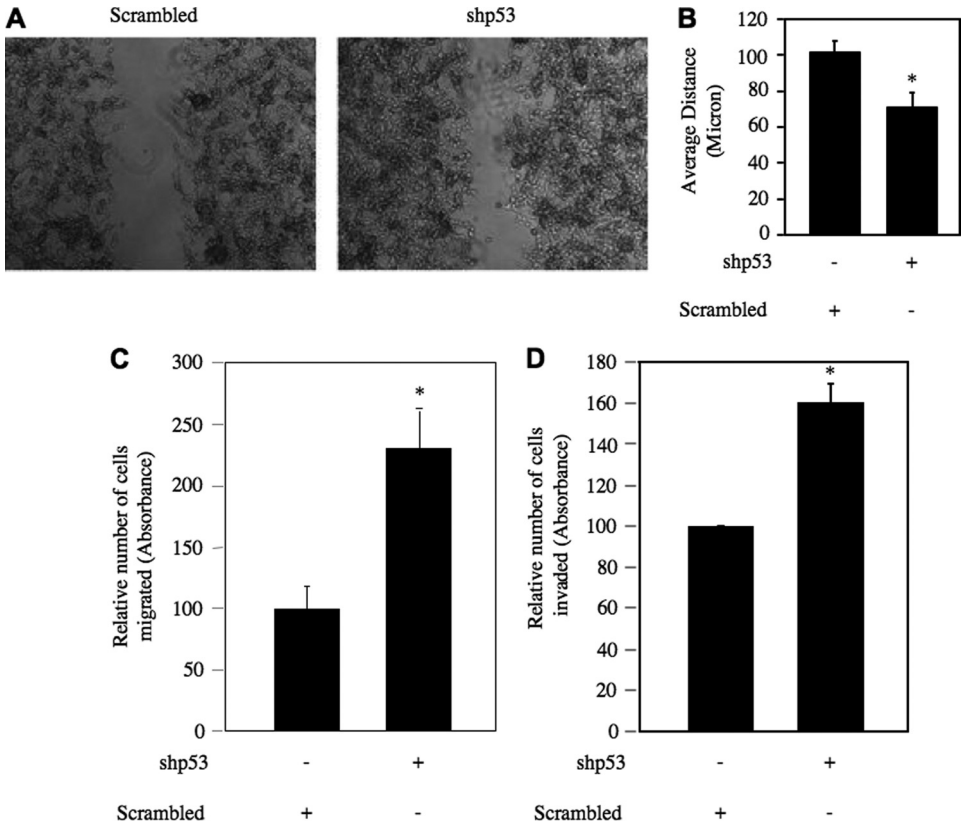

FIGURE 6. **Down-regulation of p53 increases migration and invasion of breast cancer cells.** *A,* MDA-MB-231 cells were transfected with shp53 or scramble vector. Scratch assay was performed as described under "Experimental Procedures." *B,* quantification of the scratch assay shown in *A*. The distance between the cell edges was determined by BIOQUANT as described under "Experimental Procedures." Mean ± S.E. of four independent fields is shown. *, $p = 0.002$ *versus* scramble-transfected. *C,* down-regulation of p53 inhibits migration of breast cancer cells. MDA-MB-231 cells transfected with shp53 or scramble vector were used in trans-well assay as described in the Fig. 2*E*. Mean ± S.E. of three determinations is shown. *, $p = 0.03$ *versus* scramble-transfected. *D,* down-regulation of p53 increases invasion of breast cancer cells. MDA-MB-231 cells were transfected with constructs as described in *C*. The cells were used for invasion assay with collagen-coated membrane in trans-well chambers as described in Fig. 2*H*. Mean ± S.E. of three determinations is shown. *, $p = 0.01$ *versus* scramble-transfected.

CD44 and shows positive correlation with the "bone metastasis signature" protein expression (44, 47, 48, 51–53). Our results show that statins not only suppress the expression of CD44 mRNA and protein in MDA-MB-231 cells but also they do so in other metastatic BT-20 human breast cancer cells (29) (Fig. 3, *A–C,* and supplemental Fig. S6). Additionally, simvastatin inhibited the expression of CD44 in the tumor xenografts generated by MDA-MB-231 cells (Fig. 7), which may result in reduced bone metastasis (Fig. 1). Similarly, down-regulation of CD44 in the breast tumor cells was associated with significant attenuation of migration and invasion of these cells *in vitro* (Fig. 3 and supplemental Fig. S8). These results provide the evidence for a mechanism and for a salutary role of statins in breast cancer metastasis.

CD44 represents the main receptor for hyaluronan, a non-sulfated disaccharide of D-glucuronic acid (1-β-3) and *N*-acetylglucosamine (1-β-3), although CD44 binds to other extracellular matrix proteins, including fibronectin, collagen, and osteopontin (54). The extracellular domain adjacent to the transmembrane segment of CD44 contains a variable region due to inclusion of various combinations of exons 6–15 via differential splicing. Breast cancer cells express the smallest CD44s lacking the variable exons along with different variable isoforms (CD44v) (55). Both CD44s and its various splice variants play an important role in breast cancer metastasis (35, 55). The cytoplasmic domain of all CD44 isoforms contain a 72-amino acid segment, which binds to ankyrin and ERM proteins (55). Binding of hyaluronan or other extracellular matrix ligands to the extracellular domain increases its interaction with ezrin and ankyrin to modulate the cytoskeleton necessary for cell migration/invasion and metastasis. Also, the extracellular domain of CD44 acts as a coreceptor for various receptor tyrosine kinases such as EGF receptor and VEGF receptor by directly binding to the HB-EGF and VEGF (55).

Binding of hyaluronan to CD44 has been shown to be necessary for invasion and metastasis in mammary cancer (55). Hyaluronan has been detected in the endothelium lining of the bone marrow capillaries (56). Thus, the presence of CD44 on the MDA-MB-231 cells promotes extravasation into the bone marrow cavity. Apart from this, collagen I, being the main constituent of the bone matrix and a potent ligand for CD44, can contribute to the extravasation process of the breast cancer cells. In fact, proteolytically active MMP9 is associated with CD44 on the surface of breast tumor cells (57). Therefore, MMP9-containing breast cancer cells recruited within the bone matrix may potentiate degradation of collagen I to increase bone resorption. Another important function of CD44-trapped MMP9 on the breast cancer cell surface is to produce mature

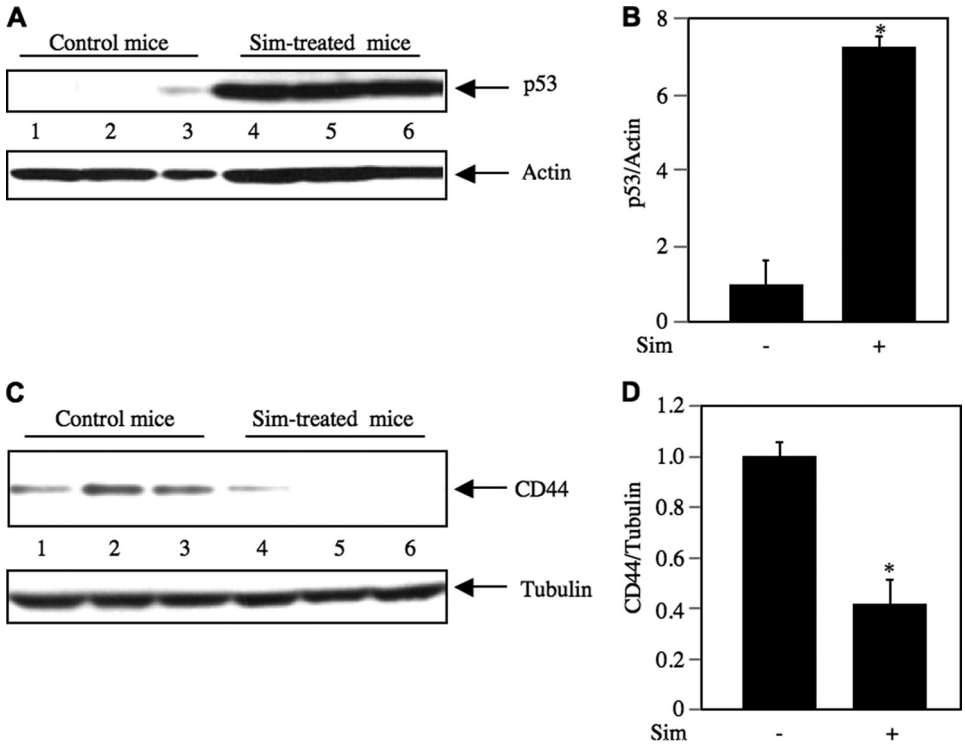

FIGURE 7. **Reciprocal expression of p53 and CD44 in tumors of mice treated with simvastatin.** *A,* lysates of tumors from control and simvastatin (Sim)-treated mice as described under "Experimental Procedures" were immunoblotted with p53 and actin (*A*) and CD44 and tubulin (*C*) antibodies. *B* shows quantification of p53 levels shown in *A*. $n = 3$; *, $p = 0.003$ *versus* control mice. *D* shows quantification of CD44 expression in *C*. $n = 3$; *, $p = 0.04$ *versus* control mice.

TGFβ, which facilitates metastasis of breast cancer cells to bone (55). Also, TGFβ increases production of parathyroid hormone-related peptide by the tumor cells to induce osteoclastic activity in the bone microenvironment. In fact, using the same mouse model of MDA-MB-231 breast cancer cell metastasis to bone used in this study, Nakamura *et al.* (58) recently showed the presence of the tumor cells positive for parathyroid hormone-related peptide facing the bone surface where the multinucleated osteoclasts are lined (58). Also in the tumor nest, CD44 immunoreactivity was observed with colocalization of hyaluronan. Our observation that simvastatin blocks the expression of CD44 in the MDA-MB-231 cells *in vitro* and in the xenografts demonstrates that this HMG-CoA reductase inhibitor may prevent metastasis of breast cancer cells to the bone (Figs. 3 and 7). This conclusion is also supported by the results showing significant prevention of the formation of osteolytic lesions in the simvastatin-treated animals (Fig. 1).

Expression of CD44 in tumor cells occurs mainly due to cytokine-mediated transcriptional up-regulation (37, 59). We also demonstrate active transcription of CD44 in MDA-MB-231 and BT-20 cells (Fig. 3, *B* and *C,* and supplemental Fig. S6, *B* and *C*). Transcription factors, including Egr1, AP1, NFκB/C/EBPβ, and Tcf-4, have been shown to up-regulate CD44 (37, 59–63). However, recent work involving analysis of various tumor tissues revealed a correlation between the expression of p53 and CD44 (64, 65). Godar *et al.* (19) recently demonstrated a negative regulatory relationship between expression of p21, a target gene for p53, and CD44 expression in breast cancer tissues of known p53 mutant status. These authors conclusively showed that wild type p53 present in an oncogene-transformed human mammary epithelial cell suppressed the expression of CD44 necessary for tumorigenesis. Also, increased expression of CD44 was observed in the basal epithelial cells of the mammary gland in p53 null mice (19). We show substantial expression of CD44 mRNA and protein in the MDA-MB-231 breast cancer cells (Fig. 3, *A* and *B*). Accordingly, the tumor xenografts also showed significant levels of CD44 protein (Fig. 7, *C* and *D*). These results apparently indicate that the low level of mutated p53 (supplemental Fig. S13) (39) present in the MDA-MB-231 cells and in tumor xenografts (Fig. 7*A*) is not active in suppressing the CD44 expression. However, we show that in MDA-MB-231 cells as well as in tumor xenografts, simvastatin significantly increased the levels of p53 (Figs. 4, *A* and *B,* and 7, *A* and *B*). Furthermore, simvastatin-stimulated increase in mutated p53 in MDA-MB-231 cells enhanced the transcription of a p53 target reporter gene and PUMA mRNA expression (Fig. 4*C* and supplemental Fig. S11). These results suggest that the mutated p53 present in the MDA-MB-231 human breast cancer cells retains transcriptional function, and when its levels are increased, it can contribute to the down-regulation of CD44 in response to simvastatin (Fig. 3, *A*–*C*). This conclusion is further supported by our observation showing increased recruitment of endogenous p53 onto the promoter of CD44 gene (Fig. 4*E*).

p53 has been shown to suppress cancer cell invasiveness (66, 67). A recent report demonstrated that early premalignant mammary cells isolated from p53 null mice showed increased mammosphere formation, indicating that p53 suppresses mammary stem cell self- renewal mainly by suppressing the "symmetric self-renewing divisions" (68). Breast cancer stem cells enriched for CD44 contribute to invasiveness (55, 69). 30%

of breast cancer patients carry p53 mutations, which are scattered throughout the coding sequence, although about 30% of them are clustered at eight hot spots (codon 175, 176, 220, 245, 248, 249, 273, and 282) in the core DNA binding domain that cause severe phenotypic consequences leading to increased tumorigenesis and invasion (66, 70, 71). However, all p53 mutations do not confer equal effects. For example, some have simple loss of function, whereas others confer either dominant negative or gain of function phenotype independently of the wild type protein and contribute to oncogenesis (72–74). The MDA-MB-231 breast cancer cell, which has the potential to metastasize to different organs, including bone, and is used in the present study, possesses homozygous missense mutation at codon 280 (R280K) (supplemental Fig. S13) (39). Although this mutation occurs in the DNA binding domain of the p53 protein, it does not completely inhibit the binding affinity for the p53 DNA element (75). Down-regulation of this mutated p53 in MDA-MB-231 cells reduced the expression of PUMA mRNA and transcription of a p53 reporter gene (Fig. 5*D* and supplemental Fig. S15). Also, our results demonstrate that inhibition of p53 expression in these breast cancer cells significantly upregulated the expression of CD44 via a transcriptional mechanism (Fig. 5, *A–C*), leading to a significant increase in cell migration and invasion (Fig. 6). Furthermore, a role of functional p53 has been reported for the inhibition of invasion of BT-20 human breast tumor cells (76). BT-20 cells have been reported to have wild type p53 (77). In contrast to these reports, mutation at codon 132 of p53 (p53K132Q) has also been demonstrated (78). However, the p53 present in the BT-20 cells showed appreciable transcriptional activity with significant effect on CD44 transcription (Fig. 12, *B* and *C*, and supplemental Fig. S6, *B* and *C*). Additionally, similar to the effect obtained with MDA-MB-231 cells, the endogenous p53 in the BT-20 cells markedly affected the invasion of these cells (supplemental Fig. S14). Together, our results provide evidence that p53 present in the MDA-MB-231 and BT-20 human breast cancer cells contribute to expression of CD44 necessary for migration and invasion of these cells.

Mouse transgenic for mutant p53A135V in a hemizygous background for endogenous wild type p53 showed accelerated tumorigenesis than the nontransgenics (79). Similarly, knock-in mouse with heterozygous p53R175H mutant allele exhibited increased incidence of metastasis (80). *In vitro*, the hot spot p53 mutants R175H, R248W, and R273H showed dominant negative effects on the wild type p53-driven transcriptional response (74). Because not all p53 mutants behave in a similar manner, we tested whether the homozygous p53R280K mutant present in the MDA-MB-231 cells or p53K132Q present in BT-20 cells behaves in a transdominant fashion. Expression of wild type p53 along with the CD44 promoter-driven luciferase reporter produced significant inhibition of CD44 transcription in both MDA-MB-231 and BT-20 breast cancer cells (supplemental Fig. S16, *A* and *B*). Importantly, expression of wild type p53 prevented the levels of CD44 protein in both these mammary tumor cells (supplemental Fig. S17). These results conclusively demonstrate that mutant p53 present in the MDA-MB-231 and BT-20 cells does not impose any dominant negative effect on the CD44 expression.

High risk patients with malignant breast cancer often carry functional p53 deficiency and continue to have relapse in other organs and bone despite effective treatment, including hormone therapy, chemotherapy, and monoclonal antibody therapy (81, 82). Our findings have therapeutic implications. Strategies to restore p53 function in tumor cells are now envisioned to prevent breast cancer metastasis. Our data provide evidence that simvastatin prevents osteolytic bone metastasis of breast cancer cells by increasing the levels of mutated p53, which is capable of repressing the expression of proinvasive CD44 protein.

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